Saskatchewan

A D F

Agriculture Development Fund

FINAL REPORT

20000290

INTESTINAL COLONIZATION IN BABY PIGS BY CLINICALLY RELEVANT E. COLI LACKING KNOWN ATTACHING FACTORS

Funded by
The Agriculture Development Fund

March 2002
Prepared by: University of Saskatchewan

SAF
Adding value to agriculture...through research and development

102-06306/

Final Report – June2002

ADF Project #20000290
Intestinal colonization in baby pigs by clinically relevant E. coli lacking known attaching factors.

Principal Investigator: D.M.Middleton[1]
Co-Investigators: J.Pritchard[1] & M.Ngeleka[2]

Department of Veterinary Pathology[1] & Prairie Diagnostic Services[2]
Western College of Veterinary Medicine
University of Saskatchewan

Rationale

The rationale for this project was to investigate strains of enterotoxigenic *E.coli* (ETEC) isolated from field cases of diarrhea in baby pigs in Saskatchewan, with no other determined cause, to identify their significance in baby piglet diarrhea. The specific strains were selected by Dr. Ngeleka based on apparent absence of fimbriae (i.e. known attachment factors).

Objectives

This project had two objectives:

i) to confirm that the selected "fimbria-negative" strains would reproduce diarrhea in baby pigs

ii) to examine microscopically colonised areas of intestine to determine the mode of attachment of these "fimbria-negative" strains.

Objective One:

The first group of eleven, colostrum-deprived, neonatal pigs acquired (source – local minimal disease herd) were randomly assigned in pairs to be inoculated with the positive control strain, the negative control broth or one of three test strains. Unfortunately these pigs were contaminated with other bacteria due to the initial procedures and the results had to be discarded due to septicaemic infections found at necropsy. A modification in the method of acquiring the piglets was made. One of the researchers physically secured each of the piglets immediately at delivery, and personally supervised their handling until reaching the animal care facility.
Two further groups of piglets were used to test the selected strains. Piglets were fed and observed at a maximum of 4-hour intervals throughout the 72-hour period of the trial. Groups were housed in separate isolation rooms. Piglets were euthanatised at the time of onset/first observation of diarrhoea.

Results:

These are listed below in Table 1, which gives the clinical signs and/or gross necropsy findings in the gut, and time of onset of clinical signs post-inoculation or the end-point of 72 hours.

Table 1: Clinical signs with time of onset and/or necropsy findings in the intestine in baby pigs inoculated with different strains of *E.coli*.

PIG #	E.COLI STRAIN # (virulence factors)	CLINICAL SIGNS/NECROPSY FINDINGS	ONSET OF CLINICAL SIGNS
13	+ ve control	Blood tinged watery scour observed	4 hours
14	+ ve control	Flaccid fluid filled intestines	4 hours
15	- ve control	Watery contents	24 hours
16	- ve control	Watery contents	24 hours
17	PD58 (AIDA, ST, EAST-1)	Blood tinged watery scour observed	25 hours
18	PD58 (AIDA, STb, EAST-1)	Blood tinged watery scour observed	19 hours
19	PD20 (AIDA, STb)	Mesocolonic edema, fluid filled intestines	15 hours
20	PD20 (AIDA, STb)	Bubbly watery contents in intestines	16 hours
21	PD71 (EAST-1)	Fluid filled intestines	72 hours
22	- ve control	Some fluid in intestines, soft formed stool	72 hours
23	- ve control	Mesocolonic edema, fluid in intestine	55 hours
24	PD71 (EAST-1)	Mesocolonic edema, soft formed stool	72 hours
26	PD149 (STb, EAST-1)	Bubbly, watery intestinal contents	17 hours
27	PD149 (STb, EAST-1)	Mesocolonic edema, blood tinged watery intestinal contents	18 hours
28	H33 (eae, EAST-1)	Mesocolonic edema, watery intestinal contents	31 hours
29	H33 (eae, EAST-1)	Watery intestinal contents	17 hours
30	PD31 (STb, LT EAST-1)	Watery intestinal contents	30 hours
31	PD31 (STb, LT EAST-1)	Very bubbly intestinal contents	19 hours

No diarrhoea was observed following inoculation of strain PD71.

Tissues were collected for histopathological examination and also for bacteriology to ascertain that the strain recovered was the same as that inoculated. Light microscopic examination showed that the different strains consistently colonized the upper colon (Figures 1 & 2: Strain D58) and it was therefore selected as the area of choice for collection of tissues for transmission electron microscopic studies in the second objective. Colonisation also occurred at various sites throughout the small intestines as would be expected with a secretory form of diarrhoea. The end point was 72 hours and piglets with no clinical signs were euthanatised at this point.

***Objective Two*:**

Strains PD149 (STb, EAST-1), PD58 (AIDA-1, STb, EAST-1) and PD31 (STb, LT, EAST-1) were selected to take forward into this experiment based on the successful reproduction of disease and the representation of various virulence factors. The negative control sterile broth was replaced with an inoculum of E.coli EAST-1 as it was felt that pigs were healthier with some bacteria to prevent colonization of the intestine with environmental clostridial bacteria. The EAST-1 strain (PD71) appeared to do this successfully in the trial for the first objective.

Piglets were euthanised and intestinal tissues were collected for histopathological examination and transmission electron microscopy (EM) at 18 and 42 hours post inoculation for each strain. The tissues for EM were fixed using two methods: standard fixation with glutaraldehyde, and a novel method of fixation using a non-aqueous fluorocarbon (FC-72) and osmium. FC-72 fixation is used by researchers (Dr D.Sims) at the Atlantic Veterinary College to study respiratory tract mucosa and this method was selected as it proved superior in preserving the glycocalyx (Figure 7 a & b) over the microvillus border of the intestinal epithelial cells, the area to which Enterotoxigenic E. coli attach.

Results:

Microscopic examination

Strain PD149

On light microscopy, strain PD149 (pathotype: STB, EAST-1) demonstrated bacterial adherence to the microvillus border in an aggregated pattern (Figure 3). In the upper colon, using transmission electron microscopy (EM), the adherence mechanism was seen to be mediated by fimbriae which attached to the microvillus border and to other bacteria (Figure 4).

Strain PD58

With strain PD58 (pathotype: AIDA-1, STb, EAST-1) bacteria were seen within a thick layer of mucus above an intact microvillus border consistent with formation of a heavy biofilm (Figure 2). EM confirmed this and also demonstrated bacteria trapped within the bacterium-mucus biofilm and in direct apposition to the microvillus border. (Figure 5)

Strain PD31

On light microscopy, strain PD31 (pathotype: STb, LT, EAST-1) also formed a biofilm-like structure which trapped the bacteria. EM confirmed bacteria within the biofilm in direct apposition to the microvillus border (Figure 6 and 7).

Agar-gel embedding and processing for EM, carried out on E.coli strain D58 (Figure 8) to allow comparison of morphology with the bacteria seen in the gut of the inoculated piglets, showed that the morphology was indeed compatible with the same organism.

PCR testing was performed on the strains isolated from each piglet to confirm that they were the same strains as those inoculated. Strains D58 and D31 were confirmed; for the strain D149 however it was also shown that the AIDA-1 factor was also present, indicating that in fact it has the same pathotype as D58 i.e. AIDA-1, STb, EAST-1.

A **cell adhesion assay** was performed outside of the scope of this study but the results are given here for completeness. The stacked brick pattern of adhesion characteristic of entero-aggregative E. coli (EAEC) (Figure 9) was seen with strain 149. while with D59 and D31 the numbers of adherent bacteria were insufficient to permit classification but were suggestive of the diffuse adherence pattern seen with diffuse adhering E.coli (DAEC) (Figures 11 & 12).

Significance

These results clearly demonstrate that the isolates of E.coli from field cases of diarrhea tested here are capable of reproducing disease when experimentally inoculated into colostrum-deprived baby pigs. They therefore need to be considered clinically relevant in cases of diarrhea where no other known causal agents can be identified. Fimbrial structures were identified in one strain (D149) but interestingly not from D58 which has the same pathotype. This would suggest that other factors not yet identified are encoding the fimbriae. Further studies are indicated to to determine if they correspond to the aggregative adherence fimbriae 1 and 2 (AAF 1 & 2) found in the entero-aggregative E.coli which is seen as an emerging cause of acute and persistent diarrhoea in children worldwide. The entrapment of the bacteria in mucus with the creation of a biofilm may allow the bacteria to cause persistent colonisation resulting in subclinical

infection and growth impairment in infants. This strain may therefore be capable of producing similar effects in piglets.

Isolate D58 demonstrated an aggregative pattern of adhesion by EM – this pattern has been noted in emerging diarrheic strains of E.coli in humans. The significance of this strain as a cause of piglet diarrhoea therefore also needs further examination.

The classification of diarrheagenic E.coli is based on toxin production and patterns of colonization. These are controlled by genetic elements which are highly mobile. This mobility potentially may lead to the generation of new hybrid strains. This is a possible explanation for the development of the strains examined in this study, as these strains do not fit into a specific category based on current classification but nonetheless are capable of producing clinical disease in neonatal pigs. A clearer understanding of such new hybrid strains will assist producers through appropriate diagnostic workup with the potential for development of vaccination programs to combat the problem within specific herds without having to resort to mass antibacterial medication.

Dissemination of information

To date:

1. Poster by Dr. J. Pritchard at the WCVM Graduate Student Poster Day in January 2002.
2. Partial results have been included in a manuscript on the "Association of E.coli Aida/STb pathotyppe with diarrhea in pigs" which has been accepted for publication in the Journal of Veterinary Diagnostic Investigation. Authors: Ngeleka,M, J.Pritchard, G.Appleyard, D.M.Middleton, and J.M.Fairbrother.
3. Isolation of a new *Escherischia coli* pathotype associated with diarrhea in Piglets. Animal Health EXPOSITOR Vol.4 Jan.2002 (published by Sask. Asgriculture & Food and Prairie Diagnostic Services. By M.Ngeleka, Microbiologist, PDS.

In preparation:

4. This work has been submitted for a poster at the 2002 Annual Meeting of the Association of American Veterinary Laboratory Diagnosticians in the fall of 2002.
5. A scientific paper is currently in preparation and will be submitted this summer to the Journal of Veterinary Diagnostic Investigation.

Lastly, this work formed the M. Vet.Sc. project for Dr J. Pritchard in the Department of Veterinary Pathology and the financial support provided by ADF made this possible.

Figures

Figure 1. Upper colon; Strain **D58**. *E. coli* bacteria (arrow) are closely associated with the mucosal surface of enterocytes. H&E. Bar = 25μm.

Figure 2. Upper colon; Strain **D58**. Streaming of bacteria (arrow) within mucous strands above enterocytes. Toluidine blue. Semi-thin section. Bar = 25μm.

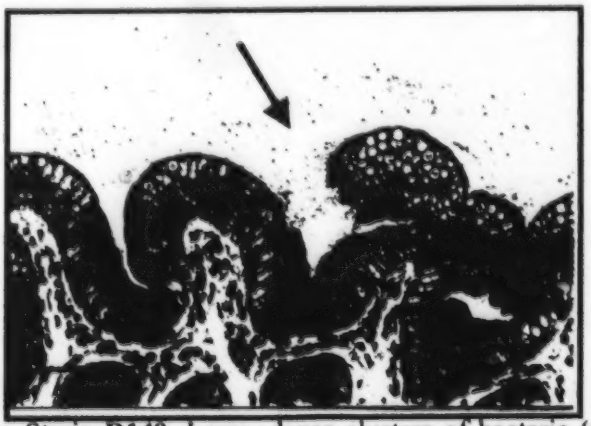

Figure 3. Upper colon; Strain **D149**. Large, dense clusters of bacteria (arrow) adjacent to the enterocytes. Toluidine Blue. Semi-thin section. Bar = 50μm.

Figure 6. Upper colon; Strain **D31.** Three bacteria are arranged linearly within a thick mucous layer in close association with the microvillus border of the upper colon (arrows). Partial halos surround each bacterium. TEM. Bar = 1.0μm

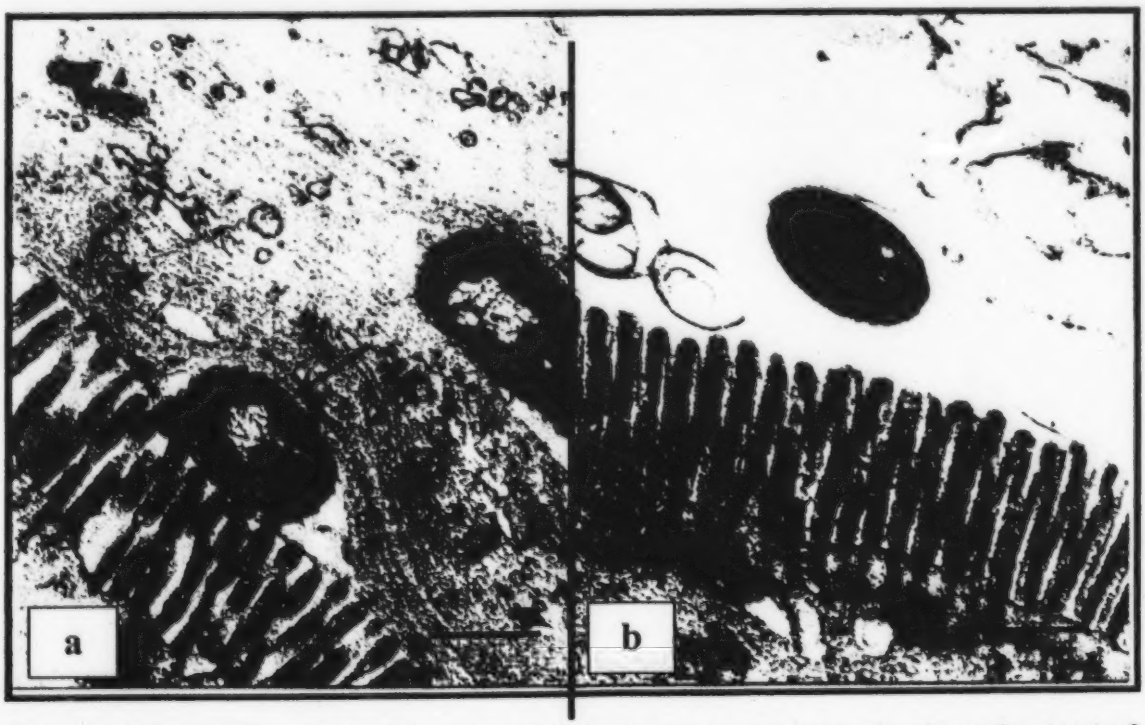

Figure 7. Upper colon; Strain **D31.** **a)** A single bacterium is seen below the glycocalyx (star) of the microvillus border. (arrow). A partial halo is present around the bacterium. Non-aqueous perfluorocarbon fixation. **b)** A single *E. coli* bacterium just above the microvillus border. Note the difference in detail of the glycocalyx in this glutaraldehyde fixed specimen. TEM Bar = 0.5μm.

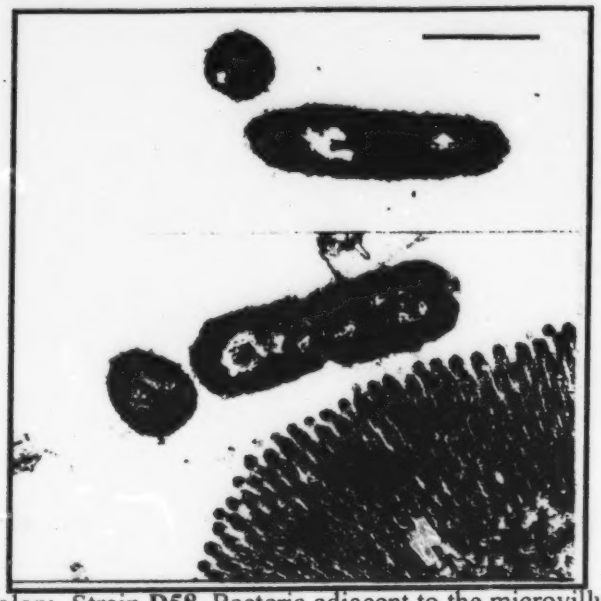

Figure 8. Upper colon; Strain **D58.** Bacteria adjacent to the microvillus border of an enterocyte. Insert: agar embedded *E. coli.* for morphological comparison of bacteria. Bar = 1.0 μm.

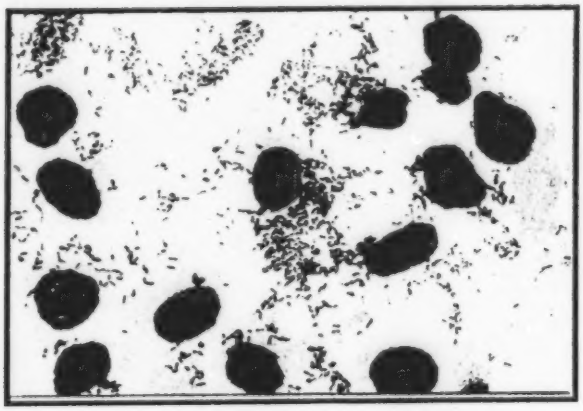

Figure 9. The classic stacked brick adhesion pattern of a positive control strain of EAEC. HEp-2 cell adhesion assay. Giemsa stain.

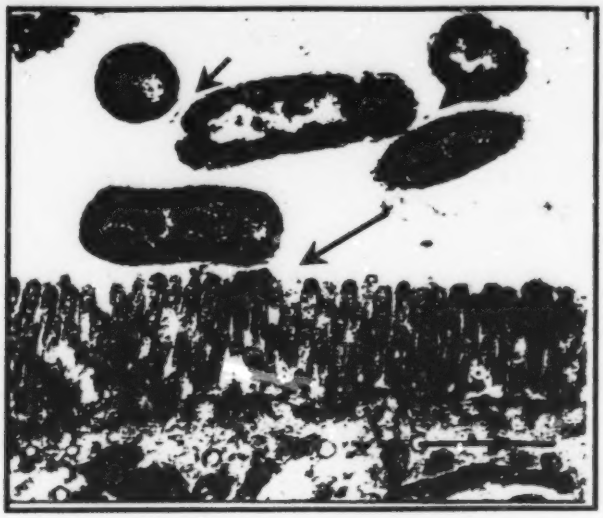

Figure 4. Upper colon; Strain **D149**. Bacteria attach to the microvillus border of the enterocytes with fine cell membrane associated fimbria (large arrow). Fimbriae also mediate interbacterial attachment. (small arrows). TEM. Bar = 1.0μm

Figure 5. Upper colon; Strain **D58** A stratified arrangement of *E. coli* bacteria within a mucous layer is seen bridging between enterocytes and in close association with the microvillus border. (large arrows) A clear halo surrounds each bacterium. Two disgorging goblet cells are present. (arrowheads) TEM. Bar = 5μm.

Figure 10. Several cells with the stacked brick adhesion pattern of *E.coli* bacteria consistent with the EAEC classification, seen with Strain D149 (STb, EAST-1). More than 40% of cells had adherent bacteria with this strain. HEp-2 cell adhesion assay. Giemsa stain.

Figure 11. Strain D31 (STb, LT, EAST-1) did not have sufficient numbers of adherent bacteria to be considered a positive test with the HEp-2 cell adhesion assay (<40%). There is one cluster of adherent E. coli in the center of the figure with a stacked brick pattern consistent with an EAEC classification. Giemsa stain.

Figure 12. Strain D58 (AIDA-1, STb, EAST-1) with a diffuse pattern of adhesion (DAEC) using the HEp-2 cell adhesion assay. There were not enough cells with bacteria adherent to be considered a positive test (<40%). Giemsa stain.